

INFLUENCE OF ACTIVATION ON THE MULTIPPOINT IMMOBILIZATION OF PENICILLIN G ACYLASE ON MACROPOROUS SILICA

**H.C.T. CARDIAS¹, C.C. GRININGER¹, H.C. TREVISAN², J.M. GUI SAN³ and
R.L.C. GIORDANO¹**

¹ Departamento de Engenharia Química, Universidade Federal de São Carlos,
V. Washington Luiz, Km 235, P. O. Box 676, 13564-210, São Carlos-SP, Brazil

² Departamento de Química Tecnologia Aplicação, Instituto de Química, (UNESP),
Araraquara-SP, Brazil ³ Instituto de Catalisis y Petroleoquímica, CSIC, Madrid, Spain

(Received: January 19, 1999; Accepted: March 30, 1999)

Abstract - Penicillin G acylase is the second most important enzyme used by industry in an immobilized form. Penicillin hydrolysis is its main application. This reaction is used to produce 6-aminopenicillanic acid (6-APA), an intermediate in the synthesis of semisynthetic antibiotics. This work aims to compare catalytic properties of different penicillin G acylase (PGA) derivatives obtained by multipoint immobilization of the enzyme on macroporous silica. Enzyme amino groups react with different aldehyde groups produced in the support using either glutaraldehyde or glyoxyl activation. In the former method, silica reacts with γ -aminopropyltriethoxysilane (γ -APTS) and glutaraldehyde; in the latter, a reaction with glycidoxypolytrimethoxysilane (GPTMS) is followed by acid hydrolysis and oxidation using sodium periodate. This work determines the influence of degree of activation, using glutaraldehyde, on immobilization parameters. PGA was immobilized on these two different supports. Maximum enzyme load, immobilized enzyme activity (derivative activity), rate of immobilization and thermal stability were checked for both cases. For glutaraldehyde activation, the results showed that 0.5% of the γ -APTS is sufficient for all the hydroxyl groups in the silica to react. They also showed that degree of activation only affects immobilization yield and reaction velocity and that reduction of the glutaraldehyde derivatives with sodium borohydride does not affect their thermal stability. In comparing the derivatives obtained using glyoxyl and glutaraldehyde activation, it was observed that the glyoxyl derivatives presented better immobilization parameters, with a maximum enzyme load of 264 IU/g silica and a half-life of 20 minutes at 60 °C.

Keywords: Penicillin G, immobilization, macroporous silica.

INTRODUCTION

After glucose isomerase penicillin G acylase (PGA) is the enzyme utilized most frequently in immobilized form by industry. Its main application is in the production of 6-amine penicillin acid (6-APA), a key intermediate in the synthesis of semisynthetic antibiotics, by N acylation of the "antibiotic nuclei" with different lateral chains. PGA's catalytic properties and stereospecificity under mild reaction conditions provide it with excellent possibilities for use as an industrial catalyst. Nevertheless, some operational disadvantages must still be solved: its fragility (liability) and high cost of recovery.

These drawbacks can be overcome by utilizing the enzyme in an immobilized form. In this way, the enzyme is in a nonsoluble form. The technology of enzyme multipoint immobilization – one molecule of enzyme is bound to several active groups on the surface support – may increase operational stability and catalytic capacity (Guisán, 1988; Pereira et al., 1997; Cardias et al., 1996). Amine groups present on the protein surface react with aldehyde groups moderately separated from the support. Different degrees of support activation imply different quantities and location of aldehyde groups that can react with protein amine groups. For each specific activation method, different distances between support surface and enzyme are established, due to the difference between aldehydic chain arms. The degree of support activation and length of the aldehydic arm may give rise to different properties of the enzyme (amine)-support (aldehyde) derivatives. The main objective of this work is to study the influence of degree of support activation on immobilization yield for the γ -APTS/glutaraldehyde method. Derivative activity and thermal stability are assessed. The properties of these derivatives are compared to those obtained by the glioxyl method (GPTMS activation).

MATERIALS AND METHODS

Materials

PGA from *E. coli* was donated by Antibióticos S.A. (Madrid, Spain); nominal activity of 520 IU/ml (one IU is defined as the quantity of enzyme necessary to hydrolyze 1 μ mol of Penicillin G 5% p/v in 0.1 M phosphate buffer pH 8.0 at 37°C). Macroporous silica medium with a pore size of 273 Å was obtained from UNESP/Araraquara (Trevisan et al., 1996) and Penicillin G from Parafarm S. A. (Brazil). Other chemicals were reagent grade.

Methods

Support activation with δ -APTS/glutaraldehyde (Cardias, et al., 1996): Silica was suspended in 0.5 % γ -aminopropyltriethoxysilane (γ -APTS) pH 3.3, 75°C with a 3mL solution/g silica ratio during 3 h of gentle agitation. The reaction mixture was filtered and washed with distilled water. The silica was then dried at 60°C, and the beads were treated with 5% glutaraldehyde in phosphate buffer pH 7.0 during 1 h, followed by exhaustive rinsing with distilled water.

Support activation with GPTMS (Pereira et al., 1997): Silica was first treated with 10% nitric acid at 25 °C during 30 minutes, followed by washing and drying. Then the beads were exposed to 5% 3-glycidyloxypropyltrimethoxysilane (GPTMS) aqueous solution pH 8.5 at 60°C during 5 h, followed by washing and drying. Epoxy groups were then hydrolyzed with 0.1 M sulfuric acid at 85°C during 2 h. The beads were washed and treated with 0.4 M sodium periodate in a 5 ml/g silica ratio during 1 h at ambient temperature, followed by washing.

Enzyme immobilization: In order to compare the activation methods utilized, a 100 mM phenyl acetic acid solution was prepared in 0.05 M phosphate buffer pH 8.0 (glutaraldehyde method) or in 0.1 M bicarbonate buffer pH 10.0 (GPTMS method). The enzyme solution, prepared at 20°C, was added to the activated wet silica in a 20 mL solution/g dry silica ratio. The slurry was gently stirred during the process. Schiff's bases were reduced using sodium borohydride (1mg H₄Br/mL solution at 20 °C during 30 minutes). The beads were rinsed with 5 mM phosphate buffer pH 7.0 and 0.01% sodium azide. To study the degree of activation with glutaraldehyde, wet silica was mixed with phosphate buffer pH 6.0, containing 100 mM of phenyl acetic acid, under gentle agitation at 20°C. Sodium borohydride was sometimes added at the end of immobilization (1 mg/ml solution at 20°C). After immobilization at pH 6.0, the silica was incubated at pH 10.0 in bicarbonate buffer at 20°C.

Amidase activity of immobilized PGA: The rate of hydrolysis of penicillin G was measured in a pHstat, based on soda consumption (10.0 ml of penicillin G 5% v/v in 10mM phosphate buffer pH 8.0 at 37°C).

Esterase activity of immobilized PGA: *p*-nitrophenol, was introduced during the hydrolysis of the synthetic substrate *p*-nitrophenyl phenylacetate ester at 25°C. Reaction was monitored in a spectrophotometer at 348 nm. Samples were taken from the reactor, using a stainless steel filter, and returned after reading.

Thermal stability: Enzymatic derivatives (20-200 mg silica/ml 0.05 M phosphate buffer pH 8.0) were incubated at 50°C (glutaraldehyde activation) or 60°C (GPTMS activation). Residual esterase activity was periodically measured.

RESULTS AND DISCUSSION

Influence of Degree of Glutaraldehyde Activation on the Immobilization of PGA on Macroporous Silica

In order to achieve supports with different numbers of aldehyde groups available to react with the enzyme (amine), the matrix was initially saturated with silane. Therefore, the degree of activation of the support could be controlled with different glutaraldehyde concentrations

Concentration of γ -APTS Solution Necessary to Achieve Total Conversion of Reactive Groups on the Silica Carrier

Three concentrations were tested: one ten times higher, one equal to and one ten times lower than that utilized in the work of Giordano and Schmidell (1992) for glucoamylase immobilization. Table 1 shows the results.

Table 1: Effect of γ -APTS concentration on immobilization yield (enzyme that disappears from the supernatant/enzyme offered to the matrix $\times 100$) and on the amidase activity of the derivative (quantity of active enzyme present on the carrier/quantity of enzyme that disappears from the supernatant $\times 100$).

Enzyme used: 10 IU of enzyme to 0.5 g silica		Immobilization yield	Recovered activity
% γ -APTS	% glutaraldehyde	(%)	(%)
0.05	2.5	55	100
0.5	2.5	86	100
5	2.5	84	100

From the results in Table 1, we may infer that a γ -APTS concentration of 0.5% and a glutaraldehyde concentration of 2.5% provide a support with maximum activation. Increasing silane concentration did not improve immobilization yield. On the other hand, a silane concentration ten times lower affected the immobilization yield, meaning

that the support's maximum activation was not achieved during the silanization step. Changes in γ -APTS concentration did not have any effect on immobilized enzyme activity. Different degrees of support activation did not affect the enzyme's conformational structure, and its catalytic capacity remained intact. We adopted a γ -APTS concentration of 0.5% as a necessary condition to guarantee that all the OH groups of the silica would be able to react with silane. In this way, it was possible to control the degree of support activation during the next preparation step: reaction with glutaraldehyde.

Influence of Glutaraldehyde Concentration on Immobilization Parameters (PGA Immobilized in the Presence of an Inhibitor – Phenyl Acetic Acid, PAA)

Figure 1 shows the variation in enzyme activity of the supernatant during the process of attachment of the enzyme to the support. All derivatives had been silanized up to saturation, but were activated with different glutaraldehyde concentrations in order to obtain distinct quantities of reactive arm chains on the support surface. We can observe that the immobilization process is faster for the Glut 2.5 and Glut 0.5 derivatives, which reach a yield greater than 90% after the first hour while the Glut 0.25 and Glut 0.05 derivatives present a 60% yield at this time. These results are kinetically consistent, since less activated supports have a lower concentration of active groups to bind to the protein.

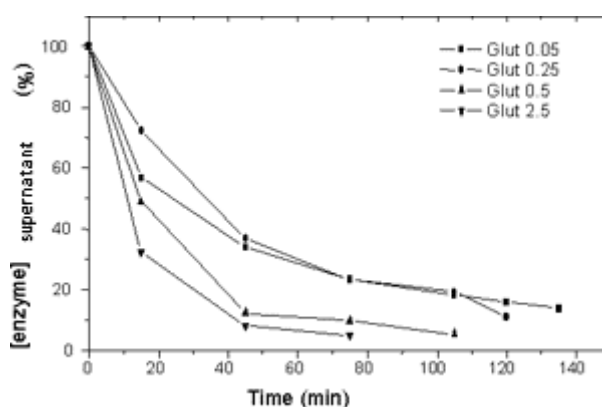


Figure 1: Immobilization of the derivatives activated with different concentrations of glutaraldehyde. Immobilization conditions: pH 6.0; T=20°C.

The reaction proceeded up to a minimum degree of immobilization of 80%. In this way, derivatives with very similar enzymatic loads were obtained facilitating further comparisons. Although the different derivatives have similar protein loads, the enzyme attached to the support at different positions due to the different degree of activation for each matrix.

Table 2 shows the activity recovered for derivatives with different degrees of activation, the pH of immobilization and whether or not they are treated with sodium borohydride. Initially, immobilization was carried out at pH 6.0. Cardias et al. (1996) had verified that this experimental condition led to the best enzyme orientation at the very beginning of the immobilization process.

Table 2: Immobilization yield and derivative activities. OE = enzyme put in contact with the matrix; IE = enzyme that disappears in the supernatant; AtAmid= amidase activity; Yield = (IE/OE)*100; Glut 0.5 = silica activated with 0.5% glutaraldehyde, immobilization at pH 6.0; Glut 0.5B = silica activated with 0.5% glutaraldehyde, immobilization at pH 6.0, final reduction with H₄Br; Glut 0.5/10 = silica activated with 0.5% glutaraldehyde, immobilization at pH 6.0 and further incubation at pH 10.0 during 3 h.

Derivative	OE (IU/g)	IE (IU/g)	Yield (%)	AtAmid (IU/g)
Glut 0.5	20	19.0	95.0	22.0
Glut 0.5B	20	19.0	95.0	21.0
Glut 0.5/10	20	19.0	95.0	16.0
Glut 0.05B	20	16.6	83.0	16.0
Glut 0.05/10	20	16.6	83.0	4.0
Glut 0.25	20	17.0	85.0	18.9
Glut 0.25/10	20	17.0	85.0	2.0

The effect of incubation at pH 10.0 was verified. This procedure increases the quantity of amino groups of the Lys-residues available to bind to the support (aldehyde). Sodium borohydride is necessary to reduce the Schiff bases formed during the reaction and to stabilize the bonds between enzyme and support.

The activity of the derivatives presented an interesting pattern: the second step of immobilization at pH 10.0 decreased this activity. This tendency is more remarkable for the less activated supports. At first glance, this decrease in activity could be explained by the improved multipoint enzyme-matrix interaction, due to the higher number of available amine groups (unprotonated) of the Lys-residues. The formation of additional bonds between enzyme and support could affect the enzyme active site and decrease its activity.

Nevertheless, this hypothesis is not feasible. The occurrence of enzyme-support multi-interactions for the poorly activated matrix is unlikely; the number of aldehyde groups available on the support is probably not high enough.

A more likely explanation for the loss of activity is that the enzyme could only be adsorbed at pH 6.0. Therefore, during incubation at pH 10.0, it would desorb from the poorly activated support. The thermal stability results in Figure 2 indicate that this hypothesis is more realistic. Additional bonds of the enzyme would imply higher derivative stability. But we can see in Figure 2 that there is no significant improvement of stability for any of the less activated supports.

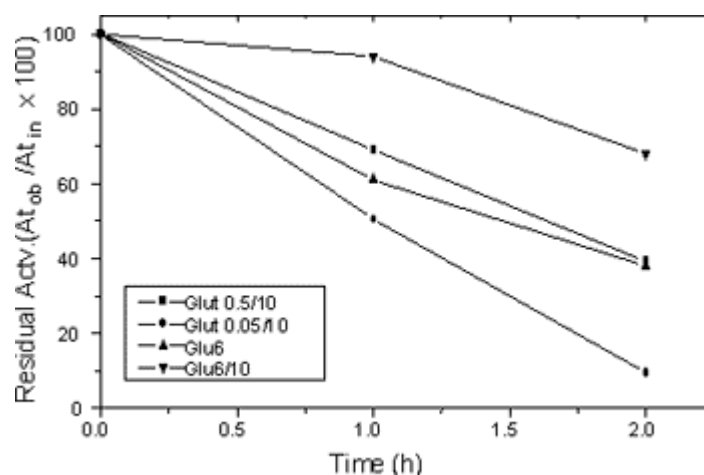


Figure 2: Thermal stability of PGA derivatives highly and poorly activated at 50°C, pH 8.0.

We can also see that reduction of the Schiff bases with sodium borohydride does not seem to affect immobilization parameters for glutaraldehyde derivatives.

Influence of Activation Method on the Immobilized Parameters of the PGA/GPTMS Activation Method

Different amounts of enzyme were offered to the highly activated glioxyl (Pereira, 1997) and glutaraldehyde supports. Silica-glutaraldehyde derivatives were immobilized at pH 8.0 as this condition is the best pH for soluble penicillin g acylase.

Immobilization Yield and Derivative Activity

The results in Table 3 show a significant decrease in immobilization yield when the enzyme concentration in the solution is increased. This behavior may be credited to a combination of diffusion and size exclusion effects at the vicinity of the pore entrance. Enzyme molecules diffusing from the bulk solution into the pores react primarily with the outer aldehydic chains – simply because those are the first chains reached by the enzyme. When highly activated silica is exposed to a large quantity of enzyme, immobilization is very fast, causing a partial or total blockage of the pore entrance. Therefore, high rates of immobilization will affect yield. The higher the rate, the higher the region of exclusion in the matrix will be.

Table 3: Immobilization yield (quantity of enzyme that disappeared from supernatant/quantity of enzyme put in contact with the matrix × 100); Immobilized enzyme (IE) and recovered activity (AtI) = actual activity/activity of the enzyme that disappeared from supernatant × 100) for different amounts of enzyme put in contact with the support (OE). Silica activated by glutaraldehyde and GPTMS methods.

Glutaraldehyde activation (γ -APTS)			Gloxyl activation (GPMTS)			Yield	
OE (IU/g)	IE (IU/g)	AtI (%)	OE (IU/g)	IE (IU/g)	AtI (%)	Glutaraldehyde	GPMTS
-	-	-	5	5	100	-	100
20	17	100	20	20	100	86	100
100	77	100	150	135	100	77	90
250	135	100	200	180	99	54	90
500	220	99	300	264	98	44	88
600	126	100	600	150	98	21	25

This phenomenon is enhanced when the support is activated with glutaraldehyde because in this case the arm chains are greater. Consequently, GPTMS derivatives present higher enzyme loads than glutaraldehyde derivatives. Results shown in Table 3 indicate that GPTMS derivatives may present an optimum enzymatic load for OE of between 300 and 600 IU/g.

When the γ -APTS/glutaraldehyde method is used with immobilization at pH 8.0, the recovered activity is close to 100%. Under this condition, a one-point enzyme-support bond is more likely to occur. At this pH, few of the Lys-amine groups present on the surface of the enzyme will be available to react. The reaction between aldehyde groups in the support and the enzyme will mostly occur with amine terminal groups of the enzyme, making unlikely the appearance of multiple bonds. With one-point bonds, the derivatives are expected to have activities near 100%, because this kind of interaction would hardly distort the active site.

When gloxyl activated the matrix, immobilization carried out at pH 10.0 led to at least a two-point interaction. Low gloxyl reactivity requires that the enzyme be multi-attached to the support. The synergistic effect of these bonds on one another will stabilize the enzyme-support interaction. At pH 10.0 it is necessary to utilize an inhibitor, which couples to the enzyme active site preventing its possible distortion. Phenyl acetic acid (PAA) plays this role, as the results in Table 3 show; the activity of these derivatives is close to 100 %.

Immobilization Rate

Figures 3 and 4 display the course of PGA immobilization on silica activated with γ -APTS and GPTMS at different enzymatic loads. For both methods of activation, immobilization rates increase as the enzyme load increases. In agreement with the previous discussion, when a highly activated support comes into contact with a high concentration of enzyme, binding to the support's outermost aldehyde groups is fast, making difficult access of other enzyme molecules to the aldehyde groups in the inner pore space.

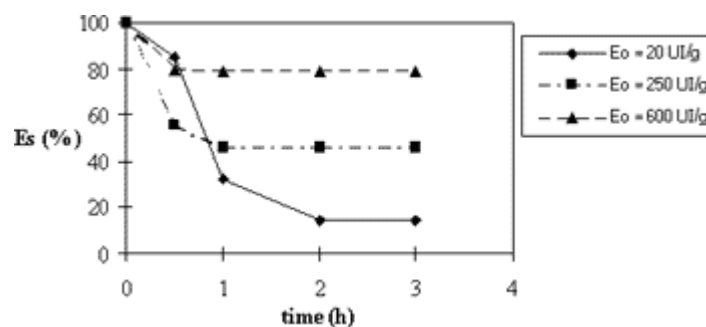


Figure 3: Immobilization time of PGA: percentage of residual enzyme in the supernatant (Es) versus time, with different quantities of enzyme (OE) put in contact with the glutaraldehyde activated silica.

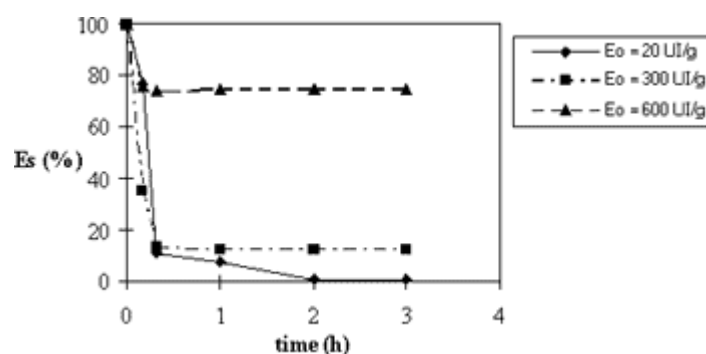


Figure 4: Immobilization time of PGA: percentage of residual enzyme in the supernatant (Es) versus time, with different quantities of enzyme (OE) put in contact with GPTMS activated silica.

In this case, the faster immobilization trade-off is a poor yield. For silica glyoxyl derivatives, immobilization rates are higher even when a low quantity of enzyme is put in contact with the matrix. This fact is due to the pH for immobilization. During the immobilization procedure at pH 8.0 with silica-glut derivatives, the enzyme has few unprotonated amino groups, which diminishes the probability of enzyme-support binding. This characteristic decreases the immobilization rate.

Thermal Stability

At 50°C half-life of γ -APTS derivatives immobilized at pH 8.0 is 30 min. γ -APTS derivatives immobilized at pH 6.0 and incubated at pH 10.0 have a half-life of 3h. However, even in this case the residual activity is 10% after 15 minutes at 60°C, while silica-glyoxyl derivatives show a half-life of 20 min under the same conditions; thus, glyoxyl derivatives have greater thermal stability than silica-glut derivatives. But it is worthy of notice that even silica-glut derivatives gained some stability when compared to soluble enzyme, which is almost instantaneously denatured at 60°C.

CONCLUSIONS

Our results permit the conclusion that a γ -APTS concentration of 0.5% is high enough to silanize all hydroxyl groups available on the surface of the macro-porous silica. Using different concentrations of glutaraldehyde may control the degree of activation. This variable affects immobilization rates, as well as interfering with the quantity of enzyme covalently bound to the support, but it has no influence on derivative thermal stability. Silica-glyoxil derivatives present higher immobilization yields and enzymatic loads. We have produced derivatives with loads up to 264 IU/g. Silica-glyoxil derivatives also present higher immobilization rates and thermal stability, with a half-life of 20 min at 60°C. Immobilized enzyme activity is not altered by any of the methods. It remains at 100% of the soluble enzyme activity.

ACKNOWLEDGEMENT

The authors thank CNPq/PADCT and CNPq/RHAE for their sponsorship of the project.

REFERENCES

- Cardias, H.T.C.; Guisán, J.M. and Giordano, R.L.C., Estudo da imobilização multipontual de penicilina G acilase em sílica ativada com glutaraldeído. Anais do 11° Simposio Nacional de Fermentações, vol. 1, pp. 60-65, São Carlos, Brazil, August 1996. [[Links](#)]
- Giordano, R.L.C. and Schmidell Netto, W., Coimobilização de enzima e microrganismo em gel de pectina para a produção de etanol a partir de amido. Atas del XIII Simposio Iberoamericano de Catalisis, pp. 643-46, Segovia, Spain, 1992. [[Links](#)]
- Guisán, J.M., Aldeyde-Agarose Gels as Activated Supports for Immobilization-Stabilization of Enzymes, Enzyme Microb. Technol., 10, pp. 375-382, 1988. [[Links](#)]
- Pereira, G.H.A.; Guisán, J.M. and Giordano, R.L.C., Multi-point Immobilization of Penicillin G Acylase on Silica-Glyoxyl: Influence of Degree of Activation, Brazilian Journal of Chemical Engineering, vol. 14, nº 4, pp. 327-332, 1997. [[Links](#)]
- Trevisan, H.C.; Contiero, J.; Hojo, O. and Monti, R., Preparação de sílica gel esférica - Livro de Resumos, TC-26, 10° Reunião Anual da SBQ, Poços de Caldas, Brazil, May 1996.